

Positive and negative regulation of JNK1 by protein kinase C and p42^{MAP kinase} in adult rat hepatocytes

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Received 2 May 1997; revised version received 29 May 1997

Abstract The role of protein kinase C (PKC) and p42^{MAP kinase} signaling in the regulation of proliferation and apoptosis was investigated in freshly isolated and primary cultured rat hepatocytes. Acute treatment of freshly isolated hepatocytes with phenylephrine and EGF caused rapid phasic activations of p42^{MAP kinase} and JNK1. Acute pre-treatment of hepatocytes with the PKC inhibitors sphingosine, chelerythrine and *bis*-indolylmaleimide abolished the ability of phenylephrine, but not EGF, to activate p42^{MAP kinase} and JNK1. Acute pre-treatments with all of the PKC inhibitors alone increased JNK1 basal activity ~2-fold. Acute treatments of primary cultures of hepatocytes with an inhibitor of MEK1 activation (PD98059) also caused inhibition of p42^{MAP kinase} and a ~2-fold activation of JNK1. These data demonstrate that PKC can function as both a proximal activator and a distal inhibitor of signaling through the JNK1/SAP kinase pathway. Treatments (4 h) of primary cultured hepatocytes with sphingosine, chelerythrine, *bis*-indolylmaleimide and PD98059 did not induce apoptosis as judged by propidium iodide staining. Similar acute treatments of HepG2 cells rapidly induced cell death. These data demonstrate that acute inhibition of either PKC or p42^{MAP kinase} function is sufficient to rapidly induce apoptosis in transformed, but not in non-transformed hepatocytes.

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Key words: p42^{MAP kinase}; JNK1; Protein kinase C; Sphingosine; *bis*-Indolylmaleimide; Chelerythrine; PD98059

1. Introduction

The mechanisms by which non-transformed cells regulate their ability to differentiate, proliferate, or undergo apoptosis are poorly understood. To better understand these processes, we have examined the regulation of signal transduction cascades in primary cultures of rat hepatocytes.

The liver is the only organ of the body which can fully regenerate its cell mass after injury [1–3]. During regeneration, hepatocytes acquire altered sensitivity/responsiveness towards multiple agonists, including a reciprocal decrease and increase, respectively, in the levels of α_1 -adrenergic and β_2 -adrenergic receptors [4–6]. We recently demonstrated that such altera-

tions in signaling lead to a decrease in the ability of epinephrine and other factors to activate protein kinase C (PKC), Raf-1, and p42/44^{MAP kinase}, and concurrently lead to an increase in the ability of epinephrine and other factors to activate JNK1 (c-Jun NH₂-terminal kinase), and p38^{SAP kinase} (p38-reactivating kinase) [6].

The JNK1/stress-activated protein (SAP) kinase cascade was initially discovered in yeast and has subsequently been characterized in mammalian cells [7–10]. Apoptotic responses have been associated with the cytotoxic lipid messengers ceramide [11,12] and sphingosine [8,13] in human myeloid leukemia cells. Activation of the SAP kinases, JNK1 and JNK2 have been directly implicated in the ceramide-related apoptosis [14,15], although the pathway linking ceramide to the SAP kinase cascade has not been completely delineated. In contrast, sphingosine-related cytotoxicity is most commonly attributed to proximal inhibition of PKC [16]. Recently we have found that, unlike ceramide, sphingosine does not engage JNK1 to promote apoptosis [17], indicating that the lethal actions of ceramide and sphingosine are subserved by distinct signaling mechanisms. In hepatocytes, however, little is known about the co-ordinated mechanisms by which alterations in receptor signaling, PKC activity, and the activities of p42^{MAP kinase} and JNK1 are altered upon liver regeneration or hepatocellular transformation. Still less is known as to how these alterations effect the actions of cytotoxic lipid second messengers and the contributions of these messengers towards regulating systems governing the initiation of apoptosis [18,19].

In the present studies, we have examined the role of PKC in the regulation of p42^{MAP kinase} and JNK1 by hormonal agonists and lipid signaling molecules, and the roles PKC and p42^{MAP kinase} play in proliferative and apoptotic signaling processes in primary isolates of hepatocytes. Our findings strongly suggest that the balance of proliferative and apoptotic signals are integrated at multiple levels, and that examination of signaling via any single pathway cannot be used to explain definitively the physiological regulation of cell survival.

2. Materials and methods

2.1. Materials

Male Sprague-Dawley rats (200 g) had access to food and water ad libitum [5]. Anti-JNK1/2 (mostly immunoprecipitating JNK1, sc-571AC) antibody was from Santa Cruz Biotechnology (Santa Cruz Biotechnologies, CA) and anti-p42^{MAP kinase} antibodies were a kind gift from Dr. M.J. Weber (University of Virginia, Charlottesville, VA). Hormones, growth factors, and propidium iodide (PI) were purchased from Sigma Chemicals (St. Louis, MO). Radiolabelled

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Abbreviations: MAP kinase, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; PKC, protein kinase C; SAP kinase, stress-activated protein kinase; AR, adrenergic receptor; EGF, epidermal growth factor

[γ - 32 P]ATP and [3 H]thymidine were from NEN. *GST-c-Jun* (a1–169) was synthesized in *Escherichia coli* and purified on GST–Sephacryl. Protein preparations of all other reagents were as described in [6,21,22]. Specific kinase domain inhibitors of protein kinase C, bis-indolyl-maleimide GF109203X [23,24], chelerythrine [18], and the *MEK1* activation inhibitor PD98059 [25] were from Calbiochem. Sphingosine was from Biomol (Plymouth Meeting, MA).

2.2. Preparation of hepatocytes

Rats were anesthetized by i.p. injection of sodium pentobarbital (50 mg/kg), and the lower thorax and abdomen was shaved to remove fur. A small (3 cm) vertical mid-line incision was made in the abdominal wall from just below the costal margin/xiphoid process. Hepatocytes were prepared by cannulation of the portal vein and collagenase perfusion of the liver. A cannula was inserted into the portal vein and two ties tightened so as to occlude the inferior vena cava, and to secure the cannula in the portal vein. Gassed Krebs-Henseleit buffer (95% air, 5% CO₂, 37°C) containing 0.3 mM EGTA (to prevent clotting), was passed through the liver (20 ml/min flow rate) to wash out erythrocytes; a second cannula was inserted into the inferior vena cava via the right atrium of the heart, and also secured with a tie, to act as a drain. Livers were washed sequentially with 200 ml of this buffer, followed by 200 ml of the same media omitting EGTA, and including 0.5 mg/ml collagenase. The liver was then removed and filtered through sterile muslin into a sterile container and washed ($\times 3$) with Krebs-Henseleit buffer. Hepatocytes were then resuspended to 5 mg wet weight/ml serum-free Krebs-Henseleit buffer containing 1.5% (w/v) Difco gelatin, and were incubated at 37°C under an atmosphere of 5% CO₂ in O₂ with continuous rotary shaking at 100 cycles/min. After a 30 min pre-incubation to stabilize basal levels of protein kinase activities (data not shown), aliquots were taken from the first portion and used immediately for experiments to determine hormonal effects on protein kinase activities [6].

2.3. Treatment of freshly isolated hepatocytes and primary cultures of hepatocytes and HepG2 cells with hormones and cell homogenization

Hormones were added to microfuge tubes containing hepatocytes (5 mg wet weight in 1 ml buffer, 1 mg total protein used per immunoprecipitate) in the absence of serum. Primary cultures of hepatocytes and HepG2 cells were cultured in 12-well plates or 20 mm dishes (2×10^5 cells) and grown in DMEM containing 10% (w/v) fetal calf serum (2 mg wet weight cells/dish, 1 mg total protein used per immunoprecipitate) and additions made to these cells after 30 min serum starvation in 5 ml of DMEM medium. Hormones were added to give final specified concentrations (see text and figure legends), vortexed/mixed to ensure complete mixing, and incubated for the specified times at 37°C in a water bath or cell culture incubator. Cells were pretreated with protein kinase inhibitors (20 min) or lipids (15 min) prior to hormonal additions. Thirty seconds prior to termination, tubes were removed from the bath, placed in a microfuge, the cells pelleted, and the supernatant removed. Plated cells were aspirated followed by immediate homogenization. Cells were homogenized in 1 ml of ice-cold buffer A (25 mM HEPES, pH 7.4 at 4°C, 5 mM EDTA, 5 mM EGTA, 5 mM benzamide, 1 mM phenylmethyl sulphonylfluoride, 1 mg/ml soybean trypsin inhibitor, 40 μ g/ml pepstatin A, 40 μ g/ml E64, 40 μ g/ml aprotinin, 1 μ M Microcystin-LR, 0.5 mM sodium orthovanadate, 0.5 mM sodium pyrophosphate, 0.05% (w/v) sodium deoxycholate, 1% (v/v) Triton X100, 0.1% (v/v) 2-mercaptoethanol), with trituration using a P1000 pipette to lyse the cells. Homogenates were stored on ice (5 min) prior to clarification by centrifugation (4°C).

2.4. Immunoprecipitations from homogenates

Fifty microliters of Protein A agarose (Ag) slurry (25 μ l bead volume) was washed twice with 1 ml of PBS containing 0.1% (v/v) Tween-20, and resuspended in 0.1 ml of the same buffer. Antibodies (2 μ g, 20 μ l) or serum (20 μ l) were added to each tube and incubated (3 h, 4°C). Clarified hepatocyte homogenates (0.5 ml, 1 mg total protein) were mixed with Protein A–Ag-conjugated antibody in duplicate using gentle agitation (2.5 h, 4°C). Protein A–Ag was recovered by centrifugation, the supernatant discarded, and washed (10 min) sequentially with 0.5 ml buffer A (twice), PBS and buffer B (25 mM HEPES, pH 7.4, 15 mM MgCl₂, 0.1 mM Na₂VO₄, 0.1% (v/v) 2-mercaptoethanol).

2.5. Assay of p42^{MAP kinase} activity

Immunoprecipitates were incubated (final volume 50 μ l) with 50 μ l of buffer B containing 0.2 mM [γ - 32 P]ATP (5000 cpm/pmol), 1 μ M Microcystin-LR, 0.5 mg/ml myelin basic protein (MBP), which initiated reactions. After 20 min, 40 μ l of the reaction mixtures were spotted onto a 2 cm circle of P81 paper (Whatman, Maidstone, UK) and immediately placed into 180 mM phosphoric acid. Papers were washed 4 times (10 min each) with phosphoric acid, and once with acetone, and 32 P-incorporation into MBP was quantified by liquid scintillation spectroscopy. Pre-immune controls were performed to ensure MBP phosphorylation was dependent upon specific immunoprecipitation of p42^{MAP kinase} [21].

2.6. Assay of JNK1 activity

Immunoprecipitates were incubated (final volume 100 μ l) with 2 μ l (10 μ g) of *GST-c-Jun* (a1–169), and reactions initiated with 98 μ l of buffer B containing 0.2 mM [γ - 32 P]ATP (5000 cpm/pmol) and 1 μ M Microcystin-LR. After 30 min, reactions were terminated with sample buffer and prepared for SDS-PAGE (10% gel) to quantify 32 P-incorporation into excised, Coomassie blue-stained *GST-c-Jun* (a1–169) bands by liquid scintillation spectroscopy. Pre-immune control assays were performed to ensure *GST-c-Jun* (a1–169) phosphorylation was dependent upon specific immunoprecipitation of JNK1 in the assay [26].

2.7. Cell culture, and primary culture and assay for DNA synthesis in hepatocytes

HepG2 cells were cultured by established procedures in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum (FCS) in 5% (v/v) CO₂. Hepatocytes isolated from sham-operated or PHX rats were cultured on rat-tail collagen (Vitrogen)-coated plastic dishes (12 $\times$ 20 mm, 2×10^5 cells, 2 mg wet weight) in William's E medium and allowed to adhere to the dish. After 2 h the medium was replaced and hepatocytes cultured in William's E medium containing 20 μ Ci of [3 H]thymidine, 1 nM dexamethasone and 100 nM insulin, in 5% (v/v) CO₂. Adrenergic drugs, hormonal treatments, and/or protein kinase inhibitors were added at the time of the media change. Cells were cultured for 48 h, after which time cells were lysed with 0.5 N NaOH and DNA precipitated with 12.5% (w/v) TCA (final). Acid precipitable material was transferred to glass fiber filters, washed with 5% (w/v) TCA, and [3 H]thymidine incorporation into DNA quantified by liquid scintillation spectrometry [27].

2.8. Transcription factor DNA binding assay

Nuclear extracts are prepared as described [5]. Oligonucleotides are 32 P-labelled with [γ - 32 P]ATP using polynucleotide kinase. Binding reaction mixtures (20 μ l) are incubated at room temperature for 45 min containing 1 ng DNA probe and 5 μ g nuclear extract in 10 mM Tris-HCl, pH 7.5, at 25°C, 40 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% (v/v) glycerol and 2 μ g poly(dI-dC), to inhibit non-specific binding in the extract. DNA–protein complexes are resolved by electrophoresis through a 4%/8% non-denaturing polyacrylamide gel containing 50 mM Tris-HCl, 0.38 M glycine and 2 mM EDTA. Gels are dried and quantified/processed using a Phosphorimager.

2.9. Data analysis

Comparison of the effects of various hormone treatments was done using 1-way analysis of variance and a 2-tailed *t*-test. Differences with a *P*-value of < 0.05 (*) and < 0.01 (**) were considered statistically significant. All bar-graph fold-values and means shown are $\pm$ SD from an average of 2–5 independent experiments (depending upon the figure) are shown.

3. Results and discussion

Freshly isolated hepatocytes were treated with phenylephrine or EGF, and p42^{MAP kinase} and JNK1 activities measured in immune-complex kinase assays (Fig. 1). Phenylephrine (Fig. 1A) and EGF (Fig. 1B) activated p42^{MAP kinase} and JNK1 with rapid phasic kinetics, with maximal activations between 4 and 10 min; maximal stimulations by phenylephrine and EGF were titrated to 10 μ M and 30 ng/ml for each hormone, respectively (data not shown). Acute (20 min) pre-

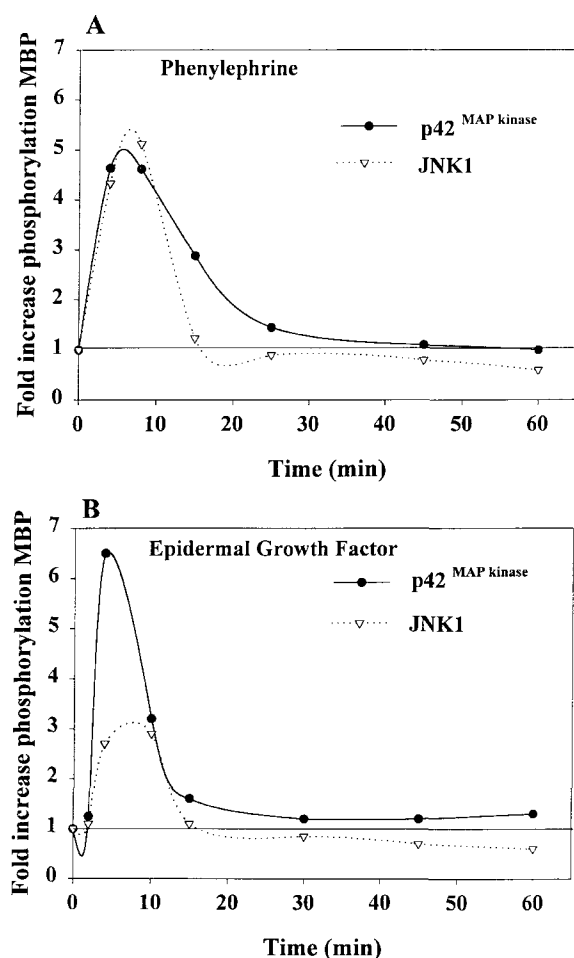
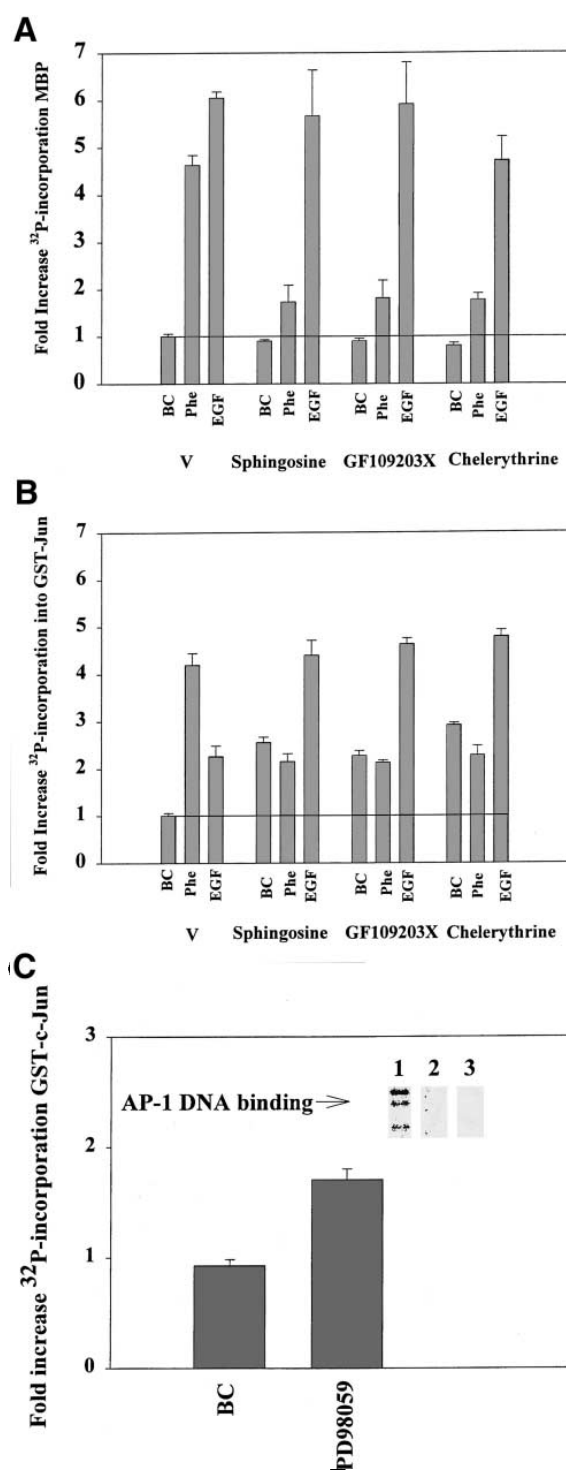


Fig. 1. Time course of activation of p42^{MAP kinase} and JNK1 by phenylephrine (10 μ M) (A) and epidermal growth factor (30 ng/ml) (B) in freshly isolated hepatocytes. Cells were treated with agonist for the indicated times at 37°C. Cells were pelleted, lysed and protein kinases specifically immunoprecipitated as described Section 2. Data are an average of duplicates from representative experiments ($n=3$) from immune-complex kinase assays and shown as -fold increases in ³²P-incorporation into substrate. Vehicle control values were p42^{MAP kinase} (25 500 $\pm$ 750 cpm) and JNK1 (950 $\pm$ 70 cpm), respectively.

treatment of cells with maximally inhibitory doses of the regulatory domain cPKC inhibitory effector, sphingosine (ti-

Fig. 2. The role of PKC in the activation of (A) p42^{MAP kinase} and (B) JNK1 in freshly isolated hepatocytes, in response to phenylephrine (phe) and epidermal growth factor (EGF) treatments. Freshly isolated hepatocytes were pre-treated (20 min, 37°C) with the maximally inhibitory doses of the PKC inhibitors sphingosine (10 μ M), bis-indolylmaleimide (10 μ M), chelerythrine (1.0 μ M), or vehicle before hormonal addition. After 20 min, cells were further treated with maximally stimulatory doses of phe (10 μ M) or EGF (30 ng/ml) for 6 min at 37°C. Cells were pelleted, lysed and protein kinases specifically immunoprecipitated as described in Section 2. Data are an average of duplicates from three experiments using immune-complex kinase assays and shown as -fold increases in ³²P-incorporation into substrate. Vehicle control values were p42^{MAP kinase} (25 500 $\pm$ 750 cpm) and JNK1 (950 $\pm$ 70 cpm), respectively. (C) Treatment of hepatocytes (20 min, 37°C) with $\pm$ PD98059 (50 μ M, +Lane 1, -Lane 2) activates JNK1 and stimulates AP-1 binding. AP-1 complex DNA binding was performed as described in Section 2 on 5 μ g of cellular lysate using non-denaturing gel electrophoresis. Lane 3, probe alone.

trated to 10 μ M), or catalytic domain *c/n/a*PKC inhibitors GF109203X (titrated to 10 μ M) and chelerythrine (titrated to 1 μ M) blunted the ability of phenylephrine, but not EGF, to activate p42^{MAP kinase} (Fig. 2A). Pre-treatment of hepatocytes with the PKC inhibitors significantly reduced the ability of phenylephrine, but not EGF, to activate JNK1 (Fig. 2B). Gq-coupled phenylephrine receptors are rapidly down-regulated in hepatocytes after isolation and primary culture [7], whereas Gq-coupled receptors for vasopressin are not. Pre-treatment of hepatocytes with the PKC inhibitors



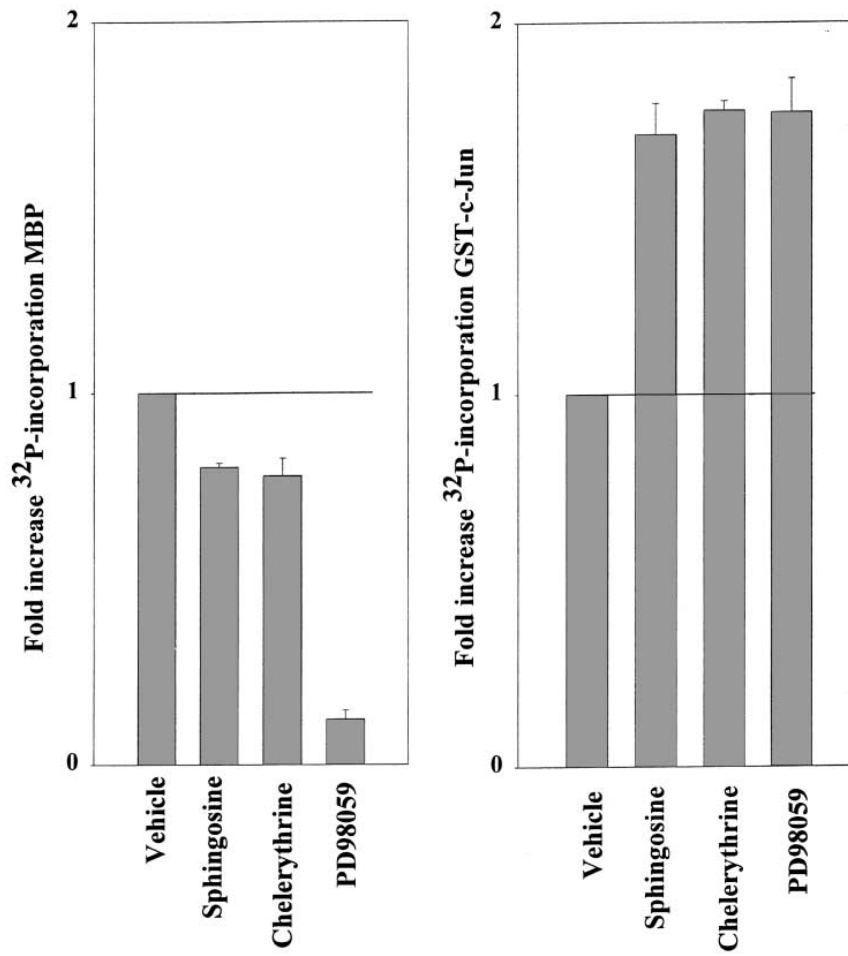


Fig. 3. The effects of sphingosine, chelerythrine, and PD98059 on the activities of (A) p42^{MAP} kinase and (B) JNK1 in primary cultures hepatocytes (20 mm well). Primary cultured hepatocytes (12 well/20 mm dishes, 2×10^5 cells) were treated for 20 min at 37°C with each of the PKC inhibitors (see Fig. 2), MEK1 inhibitor PD98059 (50 μ M), or vehicle control. Medium was aspirated, cells were lysed and protein kinases specifically immunoprecipitated as described in Section 2. Data are an average of duplicates from a representative experiment ($n=3$) from immune-complex kinase assays and shown as -fold increases in ³²P-incorporation into substrate. Vehicle control values for freshly isolated hepatocytes were p42^{MAP} kinase (20 100 ± 1000 cpm) and JNK1 (675 ± 30 cpm), respectively.

did not inhibit the ability of vasopressin (10 nM) to cause elevation of the cytosolic levels of calcium in these cells (data not shown).

Acute exposure of freshly isolated hepatocytes to maximally inhibitory doses of the PKC inhibitors sphingosine, GF109203X, or chelerythrine increased basal activity of JNK1, and reduced the basal activity of p42^{MAP} kinase (Fig. 2A,B). This disagrees with data of Beltman et al. [23] in

Rat1 fibroblasts which stated that GF109203X did not modulate JNK1/2 activity. Presumably, these differences are cell-type specific. Acute treatment of cells with a maximally inhibitory dose of the inhibitor of MEK1 activation, PD98059 (titrated to 50 μ M), also reduced basal p42^{MAP} kinase activity and increased JNK1 activity and AP-1 binding (Fig. 2C). These data suggest that inhibition of PKC, leading to inhibition of p42^{MAP} kinase function, causes an increase in the basal activity of JNK1 in adult rat hepatocytes.

Time course studies (Fig. 1) revealed that while phenylephrine caused an initial activation of JNK1, within 20–30 min the activities of these enzymes had fallen below that observed under control conditions. In contrast, the activity of p42^{MAP} kinase fell to basal by 30 min post-phenylephrine stimulation, but did not drop below this level (Fig. 1). The ability of phenylephrine to activate JNK1 was dependent upon PKC function as judged by multiple c/n/aPKC inhibitors; equally, inhibition of PKC function for 20 min promoted JNK1 activation. Since sphingosine, which inhibits c/nPKC but not aPKC isoforms, blocked the ability of phenylephrine to activate JNK1, we conclude that aPKC isoforms are not utilized by phenylephrine to stimulate JNK1 activity. Thus PKC ap-

Table 1
Effect of inhibition of PKC and p42^{MAP} kinase activities on DNA synthesis in primary cultured rat hepatocytes

Condition	-Fold [³ H]thymidine incorporation
Vehicle	1.00 $\pm$ 0.06
Chelerythrine (1 μ M)	0.11 $\pm$ 0.02*
GF109203X (10 μ M)	0.15 $\pm$ 0.05*
PD98059 (50 μ M)	0.82 $\pm$ 0.06*

Hepatocytes isolated from rats were cultured in complete medium containing 2 μ Ci of [³H]thymidine as described in Section 2. Values shown are means $\pm$ SE, expressed as fold changes compared to buffer-treated control cells ($n=17$). Significant difference from corresponding buffer control value (* $P < 0.05$). [³H]thymidine uptake in buffer-treated controls was 3200 $\pm$ 200 cpm.

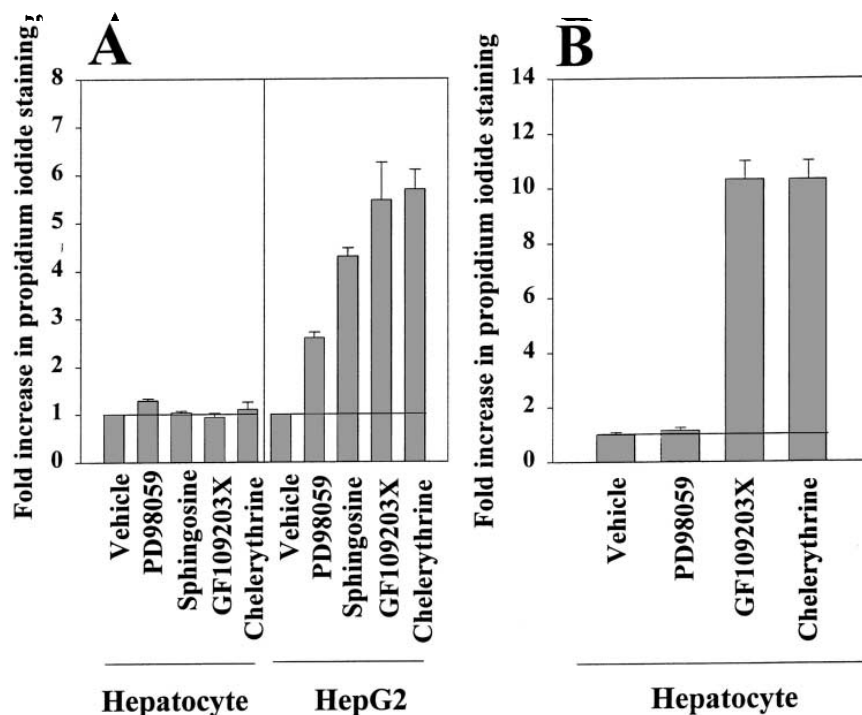


Fig. 4. Apoptosis induced by sphingosine (10 μ M), chelerythrine (1 μ M), GF109203X (10 μ M), and PD98059 (50 μ M) in primary cultures of hepatocytes and HepG2 cells after 4 h incubation. Cells were treated for 4 h with the agents indicated at 37°C (see Fig. 2). At the appropriate time, PI was added to the medium to a final concentration of 0.01% (w/v) and the cells incubated for a further 5 min at 37°C. PI staining was assessed using a video camera linked to an image quantification computer program [30]. Data are an average of five randomly selected fields on a plate from a representative experiment ($n=3$) and show the -fold increase in cells staining for PI. B: Apoptosis induced by chelerythrine (1 μ M), GF109203X (10 μ M), and PD98059 (50 μ M) in primary cultures of hepatocytes after 48 h incubation. Cells were treated for 48 h with the agents indicated at 37°C (see Fig. 2). At the appropriate time, PI was added to the medium to a final concentration of 0.01% (w/v) and the cells incubated for a further 5 min at 37°C. PI staining was assessed using a video camera linked to an image quantification computer program [30]. Data are an average of five randomly selected fields on a plate from a representative experiment ($n=3$) and show the -fold increase in cells staining for PI.

pears to play two separate roles within the same signaling pathway. First, inhibition of PKC reduced the ability of phenylephrine to activate the SAP kinase pathway suggesting acute PKC function can positively modulate this signaling cascade. Second, inhibition of PKC increased the basal SAP kinase pathway activity, suggesting that PKC function also negatively regulates these pathways.

Activation of JNK1 activity by the PKC inhibitors was unexpected, as it reveals a conserved relationship between PKC and JNK1 activities. The importance of this observation with respect to regulation of cell survival is considerable. Acute stimulation of PKC by both pharmacological (e.g. phorbol esters) and physiological (e.g. diglyceride) activators attenuates or abolishes the initiation of apoptosis by numerous ceramide-dependent stimuli [14,28,29]. Ceramide-mediated apoptosis is subject to reciprocal modulation by diglyceride and sphingoid bases in myeloid leukemia cells which respectively represent positive and negative physiological c/nPKC effector molecules [14,28,29]. The regulation of PKC function thus appears to be a key point of convergence for these mutually opposing lipid messengers, and represents a critical node for the integration of upstream lipid signals.

To extend these observations to *primary cultures* of hepatocytes, cells were acutely exposed to the PKC inhibitory lipid sphingosine, the pharmacological PKC inhibitor chelerythrine, and the MEK1 inhibitor PD98059, and p42^{MAP kinase} and JNK1 activities measured (Fig. 3). Primary cultures of hepatocytes exhibited qualitatively similar effects to those ob-

served in freshly isolated cells after treatment with either sphingosine, chelerythrine, or PD98059 (Fig. 3).

To examine the role PKC and p42^{MAP kinase} function plays in the regulation of apoptosis, primary cultures of hepatocytes and HepG2 hepatoblastoma cells were treated for 4 h with either buffer control, sphingosine, *bis*-indolylmaleimide, chelerythrine, or the specific MEK1 inhibitor PD98059. Cell survival was assessed by uptake of the vital dye PI which stains non-intact necrotic cells (reflecting 1° necrosis) or late apoptotic cells (reflecting 2° necrosis) [30].

Acute treatments (4 h) of primary cultures of hepatocytes with either sphingosine, GF109203X, or chelerythrine did not increase PI staining, but did significantly increase staining of HepG2 cells (Fig. 4A). Acute treatment of primary cultures of hepatocytes and HepG2 cells with a maximally inhibitory dose of the MEK1 inhibitor PD98059 (50 μ M) decreased basal p42^{MAP kinase} activities in both cell types by 80–90%, which was maintained for the next 4 h (data not shown). Treatment with PD98059 only caused a significant increase in PI staining in HepG2 cells (Fig. 4A). The combined effects on apoptosis of sphingosine and PD98059 co-treatment were less than additive (data not shown). Similar data were obtained when other transformed cell types, e.g. U937 myeloid leukemia cells, were treated with these compounds (data not shown). These findings indicate that transformed cell lines may be more sensitive to apoptosis, via PKC inhibition and concomitant reduction in p42^{MAP kinase} function, than are non-transformed primary cultured hepatocytes. We conclude

that the sensitivities and mechanisms by which primary cultures of hepatocytes and transformed HepG2 cells undergo apoptosis are different.

Further additional experiments then examined the abilities of chelerythrine, GF109203X, and PD98059 treatments to modulate proliferation of primary cultures of hepatocytes over a longer period of time. Maximally effective doses of chelerythrine and GF109203X which cause apoptosis in HepG2 cells but not primary cultures of hepatocytes after 4 h, reduced the ability of primary cultured hepatocytes to proliferate (Table 1) and caused apoptosis after 48 h (Fig. 4B). Treatment with PD98059, which reduced basal p42^{MAP kinase} activity by 80–90%, had a small effect on the ability of hepatocytes to proliferate (Table 1), and did not cause apoptosis in these cells (Fig. 4B). These data demonstrate that (a) inhibition of PKC in primary cultures of hepatocytes for 48 h, but not 4 h, causes apoptosis; (b) chronic inhibition of p42^{MAP kinase} by PD98059 does not dramatically reduce proliferative potential or cause apoptosis in primary cultures of hepatocytes; and (c) inhibition of p42^{MAP kinase} function in transformed hepatocytes is sufficient to cause cell death, but that inhibition of p42^{MAP kinase} in non-transformed hepatocytes does not. However, since chronic inhibition of PKC caused apoptosis in primary cultures of hepatocytes, we can dissociate inhibition of PKC function from inhibition of p42^{MAP kinase} function as the key step in causing apoptosis in non-transformed primary cultures of hepatocytes. Overall, data suggest that non-transformed primary cultures of hepatocytes are more resistant to apoptotic signals than are transformed cells, which may permit better design of chemotherapeutic treatments.

Data presented in the present report suggest that the activities of PKC, p42^{MAP kinase}, and JNK1 are in tightly regulated by multiple stimuli, and that it is the balance of regulatory signals emanating from these enzymes which determines cell fate. Our data suggests that the ability of PKC to modulate downstream signaling pathways is potentiated by cellular transformation. These mechanisms may thus represent a general signaling paradigm through which information flow along multiple signaling pathways is integrated to produce an observed physiological response.

Acknowledgements: Work was supported by the Department of Radiation Oncology (MCV) and aided by Grant IN-105V from the American Cancer Society, to P.D. who wishes to thank Dr. M.J. Weber for antibody, and Mrs. Pat Bohdan and Dr. Edward J.N. Ishac for technical assistance during these studies.

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